CHARACTERIZATION OF MICROSOMAL CYTOCHROME P450-DEPENDENT MONOOXYGENASES IN THE RAT OLFATORY MUCOSA

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Received February 9, 2005; accepted May 4, 2005

ABSTRACT:
Nasal administration of a drug ensures therapeutic action by rapid systemic absorption and/or the entry of some molecules into the brain through different routes. Many recent studies have pointed out the presence of xenobiotic-metabolizing enzymes in rat olfactory mucosa (OM). Nevertheless, very little is known about the precise identity of isoforms of cytochrome P450 (P450)-dependent monooxygenases (P450) and their metabolic function in this tissue. Therefore, we evaluated mRNA expression of 19 P450 isoforms by semiquantitative reverse transcriptase-polymerase chain reaction and measured their microsomal activity toward six model substrates. For purposes of comparison, studies were conducted on OM and the liver. Specific activities toward phenacetin, chloroxazone, and dextromethorphan are higher in OM than in the liver; those toward lauric acid and testosterone are similar in both tissues, and that toward tolbutamide is much lower in OM. There are considerable differences between the two tissues with regard to mRNA expression of P450 isoforms. Some isoforms are expressed in OM but not in the liver (CYP1A1, 2G1, 2B1, and 4B1), whereas mRNA of others (CYP2C6, 2C11, 2D2, 3A1, 3A2, and 4A1) is present only in hepatic tissue. Although expression of CYP1A2, 2A1, 2A3, 2B2, 2D1, 2D4, 2E1, and 3A9 is noticed in both tissues, there are a number of quantitative differences. On the whole, our results strongly suggest that CYP1A1, 1A2, 2A3, 2E1, 2G1, and 3A9 are among the main functional isoforms present in OM, at least regarding activities toward the six tested substrates. The implication of olfactory P450-dependent monooxygenases in toxicology, pharmacology, and physiology should be further investigated.

Nasal administration of drugs is considered to be an interesting alternative to the oral route. It allows a systemic passage through the respiratory mucosa which avoids the first hepatic passage and gives direct access to the brain through the olfactory mucosa (OM). Rapid transport to the brain may result from a paracellular passage into the cerebrospinal fluid and, more slowly, from an intracellular passage followed by axonal transport (Minn et al., 2002). Besides, the presence of drug-metabolizing enzymes in olfactory tissues is well established, especially monooxygenase-dependent activities related to the cytochrome P450 (P450) superfamily, and conjugating enzymes such as UDP-glucuronosyltransferases, glutathione S-transferases, sulfotransferases, and epoxide hydrolases (for reviews, see Thornton-Man reira et al., 2003). Only a few authors report a comparison between Olfactory P450 activities and/or expression have been studied in mice, rats, rabbits, dogs, and monkeys (Thorton-Manning and Dahl, 1997), animals commonly used for drug development. Recently, Zhuo et al. (2004) generated homozygous Cyp2g1-null mice, which do not express the specific olfactory CYP2G1. However, studies in humans are scarce (Ding and Kaminsky, 2003) because of the difficulty in obtaining olfactory tissue samples.

In rats, P450-dependent activities were described about 20 years ago in both olfactory and respiratory mucosa (Hadley and Dahl, 1982). Some studies specifically concern the formation of toxic metabolites by OM after the administration of various molecules and thus the potential role of P450 in nasal toxic events (Ding et al., 1996; Gu et al., 1998; Longo et al., 2000). Recent papers focus on the possible induction of olfactory isoforms (Genter et al., 2002; Robottom-Ferreira et al., 2003). Only a few authors report a comparison between P450 activities or expression in OM and the liver. Among them, Hadley and Dahl (1982) compared P450-dependent activities toward para-nitroanisole, aniline, aminopyrine, and hexamethylphosphor-
amidine in the OM and liver of rats. They observed that P450 activities in OM were of the same order of magnitude as or higher than those in the liver. Several P450s present in the liver were also revealed by Western blot or immunohistochemistry in OM: CYP1A1/2, CYP2B1, CYP2C, CYP2E1, CYP2J4, and CYP3A1 (Chen et al., 1992; Zhang et al., 1997; Wardlaw et al., 1998; Deshpande et al., 1999; Genter et al., 2002). CYP2A3 protein, weakly expressed in the liver, is largely expressed in olfactory tissues (Chen et al., 1992). Moreover, a specific isoform, CYP2G1, exclusively expressed in OM has been characterized by Neff et al. (1989). Published data report the detection of only four mRNAs coding for CYP2A3, 2E1, 2G1, and 2J4 in rat OM, using either Northern blot (Nef et al., 1989; Zhang et al., 1997; Longo et al., 2000) or, more recently, RT-PCR (Wang et al., 2002; Robottom-Ferreira et al., 2003).

Considering the major role of these enzymes in pharmacology and toxicology, and observing that data from the literature are often fragmentary, it seemed necessary to carry out a more exhaustive study of P450 activities and expression in olfactory tissues and to compare them with those in the liver, the main drug-metabolizing organ. To this end, we measured in the liver and OM micosomes P450 activities toward six different model substrates: phenacetin, chlorozoxazone, tolbutamide, lauric acid, dextromethorphan, and testosterone. These molecules are classically used in the evaluation of liver activities involving the CYP1A, 2E1, 2C, 4A, 2D, and 3A isoforms, respectively. Various inhibitors were tested to clarify the role of certain isoforms in the metabolism of the model substrates: ketoconazole, furafylline, α-naphthoflavone, diethyldithiocarbamate, quinine, and 5- and 8-MOP. These compounds are known to inhibit CYP3A, 1A2, 1A, 2E1, 2D, and 2A3 and 2G1, respectively. Using semiquantitative RT-PCR, a quite suitable method for intertissue comparison studies, we determined mRNA expression of 19 P450 isoforms in the OM and liver. Considering our results, and data from the literature, reporting the use of recombinant enzymes, we provide an original element for the identification of P450 isoforms involved in the metabolic activity of OM.

Materials and Methods

Chemicals. 14C-labeled substrates were purchased from Amersham Biosciences Europe (Orsay, France), except 14C-phenacitin, purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All unlabeled substrates and reference chemicals for each metabolite were purchased from Sigma, except 6-OH-chlorozoxazone, purchased from Ultrafine Chemicals Ltd. (Manchester, UK) and dextrometan ρ-d-tartrate, from MP Biomedicals (Orsay, France).

Enzymes and chemicals used for RT-PCR were purchased from Promega France SARL (Charbonnières-les-Bains, France), except Taq polymerase, purchased from Laboratories Eurobio (Les Ulis, France).

Animals. Male Wistar rats (180–200 g) were supplied by Iffa Credo (L’Arbresle, France) and housed in a room maintained at 20–22°C with a 12-h light/dark cycle. The animals received standardized chow and tap water ad libitum and were acclimatized for at least 1 week prior to experiments. The study protocol was approved by the local Animal Ethics Committee, and the research complied with the National Institutes of Health Principles of Laboratory Animal Care.

The rats were killed by decapitation. The olfactory epithelium was carefully scraped from the nasal cavity and placed into a sterile phosphate buffer (0.05 M, pH 7.4) to remove any cartilage debris. The liver was also harvested and immersed in sterile phosphate buffer. The samples of epithelium and liver were immediately frozen in liquid nitrogen and stored at −80°C until used. Microsomes were prepared from a pool of five livers and a pool of 40 olfactory epithelia as described by Gradinaru et al. (1999).

Measurement of P450-Dependent Activities. Standard incubation mixtures (500 µl, final volume) contained microsomes (0.25–2 mg of protein), 0.1 M Tris-HCl buffer, pH 7.4, 5 mM MgCl₂, and the 14C-labeled substrate or dextromethorphan at the concentration specified below. The mixtures were incubated at 37°C, for 10 to 30 min, with or without inhibitors. For inhibition studies, only one experiment was performed because of the small amount of available material. All inhibitors used (ketoconazole, furafylline, α-naphthoflavone, diethyldithiocarbamate, quinine, and 5- and 8-MOP) were dissolved in methanol; however, the percentage of methanol within the incubation medium was never higher than 1% of the total volume and did not interfere with the reaction. The incubation mixture was agitated for 5 min before starting the reaction by adding NADPH (2 mM, final concentration). Reactions were terminated by the addition of methanol under agitation and cooling on ice for 10 min. After centrifugation for 10 min at 1500g, the supernatant was collected for metabolite measurements. Product formation was determined using a Packard Liquid Chromatograph HP 1100 (Agilent Technologies France, Massy, France) coupled to on-line radiochemical detection, a Packard 150 TR Flow Scintillation Analyzer, or a fluorimetric determination with a PerkinElmer LC 240 Detector (PerkinElmer, Courtaboeuf, France).

For measurements of testosterone hydroxylation, microsomes were incubated with 200 µM 14C-testosterone (57 mCi/mmol), and the radioactive hydroxylated metabolites were separated by a combination of isotropic and solvent gradient elution. Solvent A was methanol in water (30:70 v/v), adjusted to pH 4.5 with acetic acid; solvent B was a mixture of methanol and acetonitrile (90:10 v/v), adjusted to pH 4.45 with acetic acid; solvent C was pure methanol. The initial solvent composition was 89% buffer A and 11% buffer B, maintained for 10 min following injection of each sample. A gradient elution was then used for the following 40 min until a ratio of 72% A to 28% B was obtained. This was followed by an isotropic mode for 20 min. At 60 min, elution was done with 100% C for 10 min. The flow rate was 1.5 ml/min.

The other hydroxylation activities were measured by using the following substrate concentrations: 1000 µM 14C-chlorozoxazone (57 µCi/mmoll), 50 µM 14C-tolbutamide (61 mCi/mmol), and 100 µM 14C-lauric acid (58 µCi/mL). The determination of phenacetin ß-deethylation was based on the measurement of acetaminophen production from 200 µM 14C-phenacetin (5.7 µCi/mmol). After stopping the reactions involving the different substrates (phenacetin, chlorozoxazone, tolbutamide, and lauric acid), aliquots from the supernatant were analyzed by high-performance liquid chromatography using an elution solvent gradient. Solvent D was a 0.1% solution of trifluoroacetic acid in water, whereas solvent E was pure acetonitrile. Gradient elution was used as follows: 0 to 20 min, 90% to 63% D; and 20 to 30 min, 63% to 40% D, except for lauric acid metabolites, for which the gradient was 0 to 14 min, 64% D; 14 to 18 min, 64% to 10% D; 18 to 25 min, 10% D; and 25 to 30 min, 0% D. The flow rate was 1.0 ml/min. Radioactivity was detected using on-line radiochemical detection.

Dextromethorphan demethylation was measured using a substrate concentration of 400 µM. The dextromethorphan metabolite was separated by high-performance liquid chromatography, using solvents D and E and the following elution process (flow rate, 1.0 ml/min): 0 to 20 min, 80% to 53% D; and 20 to 25 min, 53% to 30% D. The metabolite was detected by fluorimetry, with an excitation wavelength of 200 nm and an emission wavelength of 305 nm, and quantified by reference to a standard concentration.

Determination of Apparent Kinetic Parameters. We determined the apparent kinetic parameters of the biotransformation of testosterone, phenacetin, chlorozoxazone, and dextromethorphan using the method of Bertrand et al. (2000). Two incubations similar to those described above were performed for each substrate with either low or high substrate concentrations and, respectively, low or high microsomal protein concentrations (4 or 100 µM testosterone, and 0.05 or 0.5 mg/ml microsomal proteins; 1 or 100 µM phenacitin, and 0.1 or 0.5 mg/ml microsomal proteins; 5 or 500 µM chlorozoxazone, and 0.05 or 0.5 mg/ml microsomal proteins; or 1.5 or 150 µM dextromethorphan, and 0.1 or 0.5 mg/ml microsomal proteins). Aliquots of the incubation mixture were sampled at 0, 5, 15, 30, and 60 min. Residual substrate concentrations were measured at each time, and data were analyzed with WinNonlin software (Pharsight, Mountain View, CA) to obtain apparent Kₘ and Vₘₐₓ values.

RT-PCR. Total RNA was extracted according to a microscale method, using an RNAEXE kit (Laboratories Eurobio), and RNA concentration was determined by spectrophotometry. To avoid any contamination of the RNA by genomic DNA, a DNase treatment was performed using RNase-free DNase (RQI; Promega, Madison, WI). Complementary DNA was synthesized from RNA samples by mixing 1 µg of total RNA, 100 pmol of random hexamer in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM MgCl₂,
10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase, 40 units of RNase inhibitor, and a 1 mM concentration of each deoxynucleoside-5'-triphosphate in a total volume of 20 μl. Samples were incubated at 37°C for 60 min and then diluted to 100 μl with sterile diethylypyrocarbonate-treated water. The reverse transcriptase was inactivated by a 0.2 mM concentration of each deoxynucleoside-5'-triphosphate, 50 pmol of external or genomic DNA contamination.

For each isoform, comparison between mRNA expression levels in OM and liver was achieved through amplification using the appropriate specific P450 primer set of primers and the specificity of each set of primers toward liver and OM cDNA was verified by agarose gel electrophoresis using ethidium bromide for band revelation. The bands were visualized under UV light and photographed by a computer-assisted camera (Vilber Lourmat, Marne-la-Vallée, France).

**Other Assays.** Microsomal proteins were measured by the Bio-Rad Protein Assay (Laboratories Eurobio), 1.5 mM MgCl₂, 16 mM (NH₄)₂SO₄, 150 mM potassium chloride, and 10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase, without cDNA, or without reverse transcriptase) to verify the absence of external or genomic DNA contamination.

The specificity of each set of primers toward liver and OM cDNA was confirmed by sequencing the RT-PCR products obtained from the liver and olfactory mucosa, respectively (Genome Express, Meylan, France). PCR negative controls were performed on both liver and OM samples (without polymerase, without cDNA, or without reverse transcriptase) to verify the absence of external or genomic DNA contamination.

For each isofunction, comparison between mRNA expression levels in OM and liver was achieved through amplification using the appropriate specific P450 set of primers and the β-actin primers for internal standardization. Aliquots of PCR mix were sampled at 20, 25, 30, and 35 amplification cycles. The amplified products were resolved by agarose gel electrophoresis using ethidium bromide for band revelation. The bands were visualized under UV light and photographed by a computer-assisted camera (Vilber Lourmat, Marne-la-Vallée, France).

**Specific Activity**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Measured Metabolite</th>
<th>OM (n = 4)</th>
<th>Liver (n = 4)</th>
<th>Student’s t Test (P &lt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phencacetin</td>
<td>Acetaminophen</td>
<td>11,191 ± 2123</td>
<td>1341 ± 29</td>
<td>0.01</td>
</tr>
<tr>
<td>Chloroxazone</td>
<td>6-OH-Chloroxazone</td>
<td>10,070 ± 1722</td>
<td>3210 ± 415</td>
<td>0.01</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>OH-Tolbutamide</td>
<td>8 ± 0</td>
<td>115 ± 17</td>
<td>0.01</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>α-OH-Lauric acid</td>
<td>548 ± 73</td>
<td>473 ± 88</td>
<td>N.S.</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Dextromethorphan</td>
<td>2795 ± 173</td>
<td>2291 ± 203</td>
<td>0.01</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2α-OH-Testosterone</td>
<td>N.D.</td>
<td>4500 ± 837</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2β-OH-Testosterone</td>
<td>2524 ± 291</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6α-OH-Testosterone</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6β-OH-Testosterone</td>
<td>459 ± 67</td>
<td>1800 ± 231</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>7α-OH-Testosterone</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15α-OH-Testosterone</td>
<td>3864 ± 482</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15β-OH-Testosterone</td>
<td>2454 ± 239</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16α-OH-Testosterone</td>
<td>N.D.</td>
<td>5560 ± 1066</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>16β-OH-Testosterone</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Androstenedione</td>
<td>608 ± 42</td>
<td>3600 ± 1208</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1741 ± 226</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

N.D., not detectable; x, unidentified metabolite.
Results

P450-Dependent Activities. We measured the specific activities of OM and liver microsomes toward six model substrates (Table 2). The transformation rate of phenacetin to acetaminophen was 8 times higher in the OM than in the liver. Likewise, OM microsomes presented an activity toward chlorzoxazone 3 times higher than that of hepatic microsomes. Activity toward tolbutamide in the OM was much lower than that in the liver and was the lowest measured in this olfactory tissue. Concerning lauric acid, the enzymatic activity was similar in OM and liver microsomes. For dextromethorphan, activity measured in OM was slightly higher than that in the liver. Moreover, the chromatographic analysis showed that an unidentified metabolite (named x in Fig. 1) was formed by both hepatic and OM microsomes, but in different amounts. In addition, OM and the liver produced two different additional metabolites (y and z, respectively), which were not identified (Fig. 1). Lastly, testosterone was metabolized at similar rates by the microsomes of both tissues (15,460 pmol/min/mg liver protein and 11,650 pmol/min/mg OM protein). However, the hydroxy-metabolites formed in the two tissues were different (Fig. 2). 15α-, 15β-, and 2β-OH-Testosterone, androstenedione, and one unidentified metabolite were the five main products formed by OM microsomes, whereas 16α-, 2α-, and 6β-OH-testosterone and androstenedione were the major metabolites produced by liver microsomes.

Inhibition. We tested the effects of inhibitors on OM activities toward the four substrates extensively metabolized by this tissue: phenacetin, chlorzoxazone, dextromethorphan, and testosterone. Five inhibitors (ketoconazole, furafylline, α-naphthoflavone, diethyldithiocarbamate, and quinine) were tested toward only one determined activity because of their high selectivity; the two others (5- and 8-MOP), which were less selective (see Discussion), were tested toward the four activities (Table 3). Ketoconazole inhibited only 10% of the activity toward testosterone, whereas 5- and 8-MOP were more efficient (80% of inhibition). For phenacetin metabolism, almost total inhibition (94%) was obtained with α-naphthoflavone. The activity toward this substrate was decreased by 50% in the presence of furafylline, and total inhibition was obtained with 5- and 8-MOP. Addition of diethyldithiocarbamate to the incubation mixture decreased chlorzoxazone hydroxylation by 50%, whereas 5- and 8-MOP at the highest concentration gave 90% inhibition. The highest concentration of quinine decreased dextromethorphan metabolism by only 23%, whereas 5- and 8-MOP totally inhibited it.

Michaelis-Menten Apparent Kinetic Parameters. Table 4 presents apparent kinetic parameters and the efficiency of the enzyme for the four most intensively metabolized substrates in the OM. Determination of
apparent $K_m$ highlighted the fact that affinities of OM microsomes toward phenacetin and chlorzoxazone were about 10 times higher than those of liver microsomes, whereas $V_{max}$ confirmed the high activity of OM toward these substrates in comparison to the liver. For dextromethorphan and testosterone, $K_m$ and $V_{max}$ values were very similar in both tissues. Determination of the ratio $V_{max}/K_m$ suggests that the two tissues have similar metabolic capacity for testosterone and dextromethorphan and that OM metabolism is more efficient toward phenacetin and chlorzoxazone than is liver metabolism.

**mRNA Expression.** Semiquantitative RT-PCR analysis according to the number of amplification cycles allowed us to compare expression levels of mRNA coding for a specific P450 isoform in liver and OM. The two tissues presented notable differences concerning mRNA expression of the 19 tested isoforms (Fig. 3). Messenger RNA of CYP2C6, 2C11, 2D2, 3A1, 3A2, and 4A1 were not detected in liver. In contrast, those of CYP1A1, 2A3, 2B21, 2G1, and 4B1 seemed to be expressed only in OM. Messenger RNA expression of CYP1A2 and 2E1 appeared to be similar in both tissues. Finally, mRNA of CYP2A1, 2D1, and 2D4 were more strongly expressed in the liver, whereas those of CYP2B2, 2J4, and 3A9 were more strongly expressed in the OM.

**Discussion**

Previous studies on toxic metabolite formation have pointed out the high metabolic capacity of OM. Eriksson and Brittebo (1995) demonstrated that the P450 specific activity of OM microsomes toward dichlobenil is greater than that measured in the liver. Moreover, in studying the nasal toxicity of 2,6-dichlorobenzonitrile, Ding et al. (1996) observed the formation by OM of a metabolite, dihydroxy-monochlorobenzonitrile, which was not detected after hepatic metabolism of this substrate. These examples demonstrate that metabolic differences between OM and liver do exist, suggesting qualitative and/or quantitative differences in the enzymes expressed in these tissues. Our results, based on the metabolism of six model substrates, highlight significant qualitative differences between testosterone and dextromethorphan metabolites produced by OM microsomes and those produced by liver microsomes. For chlorzoxazone and phenacetin, the measured apparent $K_m$ also suggest a difference between the two tissues in the enzymes metabolizing these substrates. Lastly, our data demonstrate that tolbutamide is significantly metabolized only by liver microsomes.

Concerning the metabolism of our six model substrates, the only comparable data in the literature concern a rabbit model. The specific activity toward phenacetin measured by Ding and Coon (1990) in rabbit OM microsomes was 5 to 10 times higher than that in liver microsomes. This ratio is similar to what we report here in the rat (8 times higher). The specific activity of rabbit OM microsomes toward lauric acid was also measured by Laethem et al. (1992), but without comparison with hepatic microsomal activity. We found similar specific activities in both rat liver and OM microsomes toward this substrate, which were very close to that obtained by the above authors in rabbit OM.
In OM, we detected the mRNA coding for CYP1A1, 1A2, 2A3, 2E1, 2G1, 2J4, and 4B, the expression of which had already been described by others at the protein level (Chen et al., 1992; Zhang et al., 1997; Wardlaw et al., 1998; Deshpande et al., 1999; Genter et al., 2002) or at the mRNA level (Nef et al., 1989; Zhang et al., 1997; Longo et al., 2000; Wang et al., 2002; Robottom-Ferreira et al., 2003). In addition, we identified for the first time the mRNA coding for CYP2C6, 2C11, 3A1, 3A2, and 3A9 in OM, which has not been documented until now. Lastly, among the 19 P450 isoforms we studied, the expression of six isoforms (2B1, 2D1, 2D2, 2D4, 3A9, and 4A1) has never been examined in OM by immunoblotting. Antibodies are commercially available only for CYP2D and 4A, but we decided not to use these since we did not detect any significant mRNA expression of these isoforms in OM.

To evaluate the metabolic capacities of the P450 isoforms located in OM, we hypothesized that mRNA expression of P450 in this tissue correlates to their protein expression. Although this hypothesis may appear highly speculative, it is perfectly validated by published data for four P450 isoforms: CYP2A3, 2E1, 2G1, and 2J4 (Nef et al., 1989; Chen et al., 1992; Zhang et al., 1997; Wang et al., 2002; Robottom-Ferreira et al., 2003). Moreover, in OM we detected the mRNA corresponding to all P450 isoforms whose protein expression had been found by others except for CYP2C11. In addition, the recent characterization of rat recombinant P450 activities gave us reference data concerning their substrate specificities (Table 5) and their individual sensitivity to different inhibitors (Table 6).

It has been established that phenacetin is mainly metabolized by CYP1A1, 1A2, and 2C6. Other P450s, such as CYP2A2, 2C11, 2C13, 2E1, 3A1, and 3A2, can metabolize this substrate to a lesser degree, whereas CYP2A1 and 2B1 do not (Table 5). Using RT-PCR, we showed that CYP1A2 is expressed in a similar way in both OM and the liver. CYP1A1 is constitutively expressed in OM, whereas it is known to appear only after induction in the liver. CYP2C6 expression in OM remained undetectable after 35 cycles. Among the other P450s mentioned above, only CYP2E1 was expressed in OM, but in amounts similar to those in the liver. Therefore, the high activity displayed by OM toward phenacetin must involve other isoforms. Moreover, this activity was decreased by 50% in the presence of furafylline, an inhibitor of CYP1A2 and, to a lesser extent, of CYP2C6 (Table 6). This inhibition confirmed the involvement of CYP1A2 in phenacetin metabolism in OM but did not exclude the implication of other P450s. α-Naphthoflavone, which is an inhibitor of CYP1A1 (Tassaneeyakul et al., 1993), strongly decreased activity toward phenacetin, but again, its specificity toward other P450s is not known. According to the literature, 5- and 8-MOP are considered as CYP2G1 and 2A3 inhibitors (Gu et al., 1998) but are also efficient on CYP1A1, 2B1, 2C6, and 2C11 activities (Tassaneeyakul et al., 1993; Table 6). These compounds completely inhibited phenacetin metabolism in our OM incubations. Altogether, these data strongly suggest the involvement of CYP1A2 and 2E1, but also of CYP1A1, 2G1, and 2A3, in phenacetin metabolism in OM. This hypothesis is greatly supported by our results, which show a substantial expression of CYP2G1 and 2A3 mRNA in this tissue. Nevertheless, we cannot exclude the possibility that other P450s are involved in this activity, for instance, CYP3A9, which is more strongly expressed in OM than in the liver. However, there are no data available concerning the possible activity of this isoform toward phenacetin.

Data from the literature indicate that chlorzoxazone is a preferential substrate of CYP2E1, but it can also be metabolized by CYP1A1, CYP1A2, CYP2C11, CYP3A1, 3A2, and 3A9. Antibodies raised against CYP2A (Su et al., 1996) or CYP2B (Wardlaw et al., 1998) have been used in OM, but their lack of specificity precluded accurate P450 identification. Even after a 35-cycle amplification, we did not observe any expression of mRNA coding for CYP2C6, 2C11, 2D2, 3A1, 3A2, and 4A1 in OM, although Western blot experiments showed that CYP2C11 and 3A2 proteins were present in very low quantities in OM (Deshpande et al., 1999; Genter et al., 2002). The conflicting results concerning CYP3A may be explained by the observations of Aiba et al. (2003): their work demonstrated the existence of an immunological cross-reaction between CYP3A2 and CYP3A9; the mRNA of the latter was clearly detected in OM in the present study. Besides, the absence of expression of CYP2C6, 2D2, 3A1, and 4A1 in OM has not been documented until now. Lastly, among the 19 P450 isoforms we studied, the expression of six isoforms (2B1, 2D1, 2D2, 2D4, 3A9, and 4A1) has never been examined in OM by immunoblotting. Antibodies are commercially available only for
Activities were calculated from the measurement of the residual substrate concentrations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Olfactory Mucosa</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (µmol/min/mg microsomal protein)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.95</td>
<td>$9.92 \times 10^{-3}$</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>3.04</td>
<td>$9.50 \times 10^{-3}$</td>
</tr>
<tr>
<td>Chlorzoxazone</td>
<td>18.7</td>
<td>$9.94 \times 10^{-3}$</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>6.11</td>
<td>$1.49 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

**TABLE 4**

Determination of the apparent kinetic parameters ($K_m$, $V_{max}$) and efficiency of the P450 for activities toward four model substrates in rat olfactory mucosa and hepatic microsomes.

inhibition (80%) was observed with OM microsomes, whereas inhibition was less than 10% with those of the liver (data not shown). On the whole, our results strongly suggest that CYP1A1, 1A2, 2A3, 2E1, 2G1, and 3A9 metabolize phenacetin and chlorzoxazone in OM, whereas certain P450s are more specifically involved in the liver (mainly, CYP1A2 for phenacetin and CYP2E1 for chlorzoxazone).

The CYP2C family (2C6 or 2C11) is responsible for the hepatic metabolism of tolbutamide in rats (Azuma et al., 1999; Matsunaga et al., 2001). We found much weaker tolbutamide hydroxylation activity in OM microsomes than in hepatic microsomes; among the tested substrates, tolbutamide was the least metabolized molecule in OM. This difference in the activities displayed by the two tissues probably results from the absence of detectable CYP2C11 and 2C6 expression in OM, whereas their mRNAs are significantly expressed in the liver.

CYP2D2 is known to be the main P450 isoform involved in dextromethorphan metabolism. Most other tested recombinant P450s, i.e., CYP1A2, 2A1, 2A2, 2B1, 2C6, 2C11, and 2E1, do not display any affinity toward this substrate or very weakly participate in its metabolism (CYP2C13, 2D1, 3A1, and 3A2) (Table 5). We showed that the hepatic isoforms belonging to the CYP2D family were either weakly (2D1 and 2D4) or not at all (CYP2D2) expressed in OM. Similarly, we did not find either CYP3A1 or 3A2 expression in OM. However, we did observe dextromethorphan metabolism of the same order of magnitude in OM and the liver. In our assays, quinine decreased dextromethorphan metabolism by 23%. This molecule inhibited CYP2D2 and, to a lesser extent, CYP2C6 and 2C11 (Table 6), but these isoforms were not expressed in OM. Unfortunately, there are no available data concerning quinine inhibition capacity toward other recombinant P450s. Finally, we showed that 5- and 8-MOP totally inhibited OM activity toward dextromethorphan.

Previous reports mentioned above and our present data thus suggest that CYP2G1, 2A3, and 1A1 may be involved in dextromethorphan metabolism in OM. Concerning this substrate, we also pointed out quantitative and qualitative differences in the metabolites formed by OM and liver microsomes. Indeed, one of the metabolites (x) from dextromethorphan is not observed in this tissue (data not shown). CYP4A9 in dextromethorphan metabolism is thus possible but remains to be demonstrated.

CYP4A1 and, to a lesser extent, CYP4A2 and 4A3 are mainly involved in $\omega$-hydroxylation of lauric acid (Table 5). This substrate can also undergo $\omega$-1-hydroxylation, preferentially carried out by CYP2E1 (Clarke et al., 1994). In the present study, CYP4A1 mRNA was only found in the liver. Conversely, CYP2E1 mRNA is significantly expressed in OM, and the presence of the corresponding isoform could explain the lauric acid $\omega$-1-hydroxylation we also observed in this tissue (data not shown). CYP4B1 can also metabolize...
lauric acid to ω-1 and ω-OH derivates in the rabbit (Muerhoff et al., 1989). It is expressed in rat lung, but not in the liver (Gasser and Philpot, 1989). Since its mRNA is expressed in OM, CYP4B1 could be mainly responsible for ω-hydroxylation of lauric acid in this tissue and, also, for ω-1-hydroxylation in association with CYP2E1. There are no data available concerning the involvement of other P450 isoforms in lauric acid hydroxylation.

Testosterone biotransformation in the liver involves various P450s, including CYP3A and 2C, and leads to the formation of numerous metabolites (Table 5). However, the profile of metabolites obtained with our OM microsomes was very different from that obtained with liver microsomes. We observed the formation of the following metabolites by OM: 2α-, 15α-, and 15β-OH-testosterone. It is well established that these metabolites can be produced from testosterone by P450 isoforms other than hepatic isoforms, such as CYP2A3 (Table 5), which is strongly expressed in OM. The liver and OM only produce two common metabolites, 6α-OH-testosterone and androstenedione. CYP2A3 cannot form these metabolites, whereas CYP3A1, 3A2, and 2C11 can produce them (Table 5). We were unable to detect expression of the three last isoforms in OM. In contrast, CYP3A9, whose mRNA is expressed in OM, is known to metabolize testosterone, producing 2β- and 6β-OH derivates (Table 5). Androstenedione, formed in smaller quantities by OM microsomes than by those of the liver, can be produced by CYP2B, whose expression was also noticed in OM. We showed that ketoconazole weakly decreased testosterone metabolism in OM. This compound inhibited CYP3A1 and 3A2 (Table 6), which are not expressed in OM, but its action on CYP3A9 is not known. In contrast, 5- and 8-MOP efficiently inhibited OM activity toward this substrate. This inhibition might concern CYP2A3, 2B, and, possibly, 2G1; the mRNAs of all three are present in this tissue. Finally, the possible role of CYP2J4 in testosterone metabolism appears very limited (Zhang et al., 1997). Altogether, our data are consistent with the possible implication of CYP2G1, 2A3, 2B, and 3A9 isoforms in OM metabolism of testosterone.

We noticed substantial mRNA expression of CYP2B21 in OM. The presence of this mRNA had only been found in the esophagus and not in the liver by Brookman-Amissah et al. (2001). However, no data are available concerning activities of the corresponding enzyme toward our model substrates. Its metabolic role in OM thus requires further investigation.

In the present work, we studied mRNA expression of a large number
of P450-dependent monoxygenases in rat OM, and we measured the activity displayed by this tissue toward a series of substrates of these monoxygenases. A comparison of our experimental data with those from other related works, especially those concerning the catalytic activity of recombinant isoforms, strongly suggests that CYPIA1, 1A2, 2A3, 2E1, 2G1, and 3A9 are among the main functional P450s in OM, at least concerning activities toward the six tested substrates. The isoforms expressed in this tissue constitute an enzymatic P450-dependent system, which is very different from the hepatic one.

Considering the role of P450-dependent monoxygenases in the detoxification of many xenobiotics, the differences we noticed in mRNA expression and in measured activities toward different substrates may contribute to tissue-specific toxicity events. Yet, these monoxygenases participate in the detoxification of numerous molecules, and their presence in OM could protect the brain, especially from toxic substances, by decreasing the direct nose-brain passage. Furthermore, these proteins frequently display strong enzymatic activities in OM, and in some cases (phenacetin and chlorzoxazone), the activities are significantly stronger than those measured in the liver. Even if the hepatic tissue mass is considerably greater than that of olfactory tissue, the possible role of olfactory metabolism in drug bioavailability should be considered, particularly for nasally administered molecules. In addition, Nef et al. (1989) and Ding et al. (1991) suggested that CYP2G1 could be involved in the olfactory process, especially in sensory and functional regulation. The functional role of CYP2G1 in olfactory epithelium is an interesting topic that needs to be further considered

Acknowledgments. We gratefully acknowledge the technical assistance of Catherine Potin and also thank Nathalie Hanet for critical review of the manuscript.

References